Occurrence of alginate gene sequences among members of the pseudomonad rRNA homology groups I–IV *

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1. SUMMARY

Total genomic DNA of 13 pseudomonads representing rRNA homology groups I–IV were screened for sequences homologous to four *Pseudomonas aeruginosa* alginate (*alg*) genes by Southern hybridization. Biotinylated probes for three structural genes (*algA, algC* and *algD*) and one regulatory gene (*algR1*) were prepared. Genomic DNA of strains representing group I (*P. syringae* pv. *glycinea, P. viridiflava* and *P. corrugata*) hybridized with all four gene probes. Hybridizing fragments were of differing sizes, indicating that evolutionary divergence among group I members has occurred. *P. corrugata* has not been reported to synthesize alginate. Genomic DNA from representatives of groups II–IV gave no or very weak hybridization with the probes except for *algC*. This study indicates that the ability to produce alginic acid as an exopolysaccharide among the pseudomonads is restricted to members of rRNA homology group I in agreement with earlier physiological studies.

2. INTRODUCTION

In their natural environments most bacteria are enveloped by a coating of high molecular mass exopolysaccharide [1]. Studies in our laboratory [2] and in others [3,4] have indicated that many fluorescent pseudomonads produce alginic acid as an exopolysaccharide. Alginic acid is also produced by *Azotobacter* species [5,6]. Alginate has been proposed to be a virulence factor for phytopathogenic fluorescent pseudomonads [2,7].

Alginic acids are a group of structurally related polysaccharides composed of a linear backbone of β-1,4-linked D-mannuronic acid and its C-5 epimer L-guluronic acid in varying ratios [8]. They are currently isolated from brown seaweeds for a variety of commercial applications such as gelling and thickening agents [9]. However, bacte-

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* Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

rial alginate differs from algal alginate due to the presence of acetyl groups on the mannuronate residues [10].

Much research has been done both on the enzymatic and molecular aspects of alginic acid production by *Pseudomonas aeruginosa* on account of the ability of mucoid variants of this bacterium to cause serious chronic lung infections in cystic fibrosis patients. Several structural, as well as regulatory, genes have been cloned, and the functions of several gene products determined (for a review see [11]). The cloned genes and the corresponding biosynthetic enzymes involved in the pathway to alginate synthesis are shown in Fig. 1. Gene *algA* is unusual in that it encodes two enzymatic activities (phosphomannose isomerase and GDP-mannose pyrophosphorylase) that do not occur immediately adjacent to each other in the pathway. The first three enzymes of the pathway are involved in general carbohydrate metaholism and are found in many bacteria. In contrast, GDP-mannose dehydrogenase encoded by *algD* is thought to be specific for alginate synthesis [12]. The *algD* gene is transcriptionally activated in mucoid strains. One positive regulatory gene, *algR1*, is a member of a class of environmentally responsive two-component regulatory genes with an effector function [13]. The gene encoding for the sensor component has not yet been identified.

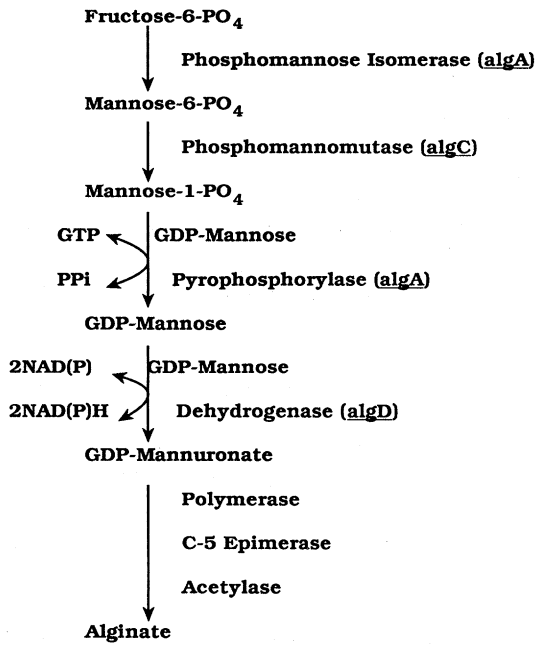

Fig. 1. Biosynthetic pathway for alginate production in *P. aeruginosa*. Cloned genes encoding for the enzymes are shown in parentheses.

Table 1

Bacterial strains used in this study

Bacterium	rRNA Homology group [a]	Alginate production	Host of origin	Source [b]
Pseudomonas corrugata 388	I	–	*Medicago sativa*	F.L. Lukezic
P. syringae pv. *glycinea* NCPPB 2159	I	+	Not known	NCPPB
P. viridiflava 679	I	+	*Medicago sativa*	F.L. Lukezic
P. viridiflava ATCC 13223	I	+	*Phaseolus coccineus*	ATCC
P. viridiflava TU-04–2A	I	+	*Lycopersicon esculentum*	C.H. Liao
P. andropogenis 27	II	–	*Cicer arietinum*	F.L. Caruso
P. cepacia 376	II	–	Not known	F.L. Lukezic
P. solanacearum K60	II	–	*Lycopersicon esculentum*	L. Sequeira
P. gladioli P32	II	–	*Asplenium nidus*	A.R. Chase
P. acidovorans ATCC 15668	III	–	–	ATCC
P. avenae VS-1	III	–	*Paspalum urvillei*	R.D. Gitaitis
P. testosteroni ATCC 11996	III	–	–	ATCC
P. diminuta ATCC 11568	IV	–	–	ATCC

[a] Homology groups based on the reports of DeVos et al. [32] and Palleroni [23].

[b] ATCC = American Type Culture Collection, Rockville, MD, USA; NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, UK.

In a previous study of the distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-*Azomonas-Azotobacter* lineage of superfamily B procaryotes *algA, algC, algD* and *algR1* were used as probes [14]. Only group I pseudomonads (except for the nonfluorescent subgroup Ia representative *P. stutzeri*) and *A. vinelandii* contained sequences homologous to all four *P. aeruginosa alg* genes used as probes. The *P. stutzeri* strain examined did not contain sequences homologous to *algD*. In that study pseudomonads belonging to groups II–IV were not studied. We prepared similar alginate gene probes and by Southern hybridization analysis determined the distribution of homologous sequences among *Pseudomonas* rRNA homology groups II–IV as well as for an additional representative of the non-fluorescent subgroup Ia.

3. MATERIALS AND METHODS

3.1. Bacterial strains

The bacterial strains used in this study are listed in Table 1. Short-term maintenance was on plates of Difco *Pseudomonas* agar F stored at 4°C. Long-term maintenance was as suspensions in trypticase soybroth (BRL) plus 15% glycerol stored at −80°C.

3.2. DNA isolation, purification and probe preparation

Genomic DNA was prepared according to a standard protocol for large scale genomic DNA preparation [14]. The CsCl gradient centrifugation step was omitted and the samples were subjected to digestion with RNase (20 μg/ml) [14]. Total DNA (3 μg) was digested with appropriate restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, MD) and the fragments were separated by electrophoresis on 0.8% agarose gels (11 × 14 cm) using 1 × TAE buffer [15]. Gels were then blotted as described below. Total genomic DNA of *P. syringae* pv. *glycinea* strain NCPPB 2159 was included as a positive control as it was previously reported to contain gene fragments homologous to the alginate genes included in this study [16].

Plasmid DNA for probe preparation was isolated from *E. coli* strains harboring plasmids pAD4033 (*algA*), pNZ15 (*algC*), pVD211 (*algD*) and pKS30 (*algR1*) containing alginate genes cloned from *P. aeruginosa* (kindly supplied by N.A. Zielinski and A.M. Chakrabarty). Plasmids were amplified with chloramphenicol [15] and crude lysates obtained by alkaline lysis [14]. Plasmid DNA was purified by polyethylene glycol precipitation [14], subjected to digestion with the appropriate restriction endonucleases, the DNA fragments separated on agarose gels and stained with ethidium bromide, and the target DNA fragments excised from the gels. Target DNA was electroeluted and then concentrated and purified using an Elutip-d column (Scleicher and Schuell Inc., Keene, NH) [14]. The concentration of purified target DNA was determined by ethidium bromide dot quantitation [14]. The DNA probes were labelled with biotin-7-dATP by use of a nick translation kit used according to the manufacturer's instructions (Bethesda Research Laboratories).

3.3. Southern blot hydridization

Three μg of restriction enzyme digested genomic DNA per bacterium was transferred to supported nitrocellulose (Bethesda Research Laboratories) according to the method of Southern [17] as described by Ausubel et al. [14]. Hybridization of the biotinylated DNA probes at high stringency (final washes with 0.16 × SSC/0.1% SDS at 50°C) was done using a BluGene nonradioactive nucleic acid detection system according to the manufacturer's instructions (Bethesda Research Laboratories) except that hybridization was allowed to continue for two days at 42°C rather than overnight.

4. RESULTS

A 2.0 kb *Bam*HI-*Sst*I DNA fragment containing the *P. aeruginosa algA* gene was cut from the plasmid pAD4033 [18] and used as a probe for the presence for homologous sequences in *Bam*HI-*Sst*I digested genomic DNA that may encode for PMI-GMP enzyme activities. This

fragment contains most of the *algA* coding sequence as well as about 40 base pairs upstream of the translational start site [18]. Hybridizing fragments were observed for the five representatives of rRNA homology group I with one fragment per strain (Fig. 2). These fragments were all of differing molecular size. Group II representatives *P. cepacia* strain 376 and *P. gladioli* strain P32 showed one or two more weakly hybridizing fragments, respectively. Hybridizing fragments were not found for the other bacteria.

A 2.6 kb *Hin*dIII-*Sst*I DNA fragment containing the *P. aeruginosa algC* gene was cut from plasmid pNZ15 [19] and used as a probe for homologous sequences in *Hin*dIII-*Sst*I digested genomic DNA that may encode for PMM enzyme activity. This fragment contains the *algC* promoter and coding region [19]. The most strongly hybridizing fragments using the *algC* probe were seen for group I strains *P. viridiflava* TU-04-2A and *P. corrugata* 388 with the other three group I representatives showing less strongly hybridizing

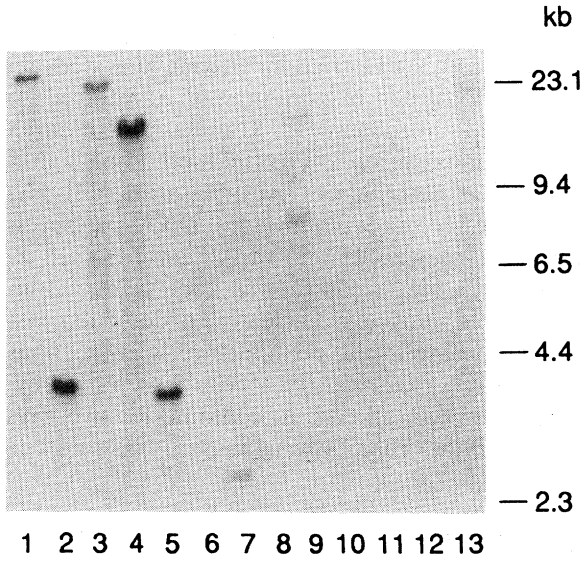

Fig. 2. Hybridization of the *P. aeruginosa algA* gene probe with *Bam*HI-*Sst*I digested bacterial genomic DNA. Lanes: 1, *P. syringae* pv. *glycinea* NCPPB 2159; 2, *P. viridiflava* 679; 3, *P. viridiflava* ATCC 13223; 4, *P. viridiflava* TU-04-2A; 5, *P. corrugata* 388; 6, *P. andropogenis* 27; 7, *P. cepacia* 376; 8, *P. solanacearum* K60; 9, *P. gladioli* P32; 10, *P. testosteroni* ATCC 11996; 11, *P. acidovorans* ATCC 15668; 12, *P. avenae* VS-1; 13, *P. diminuta* ATCC 11568.

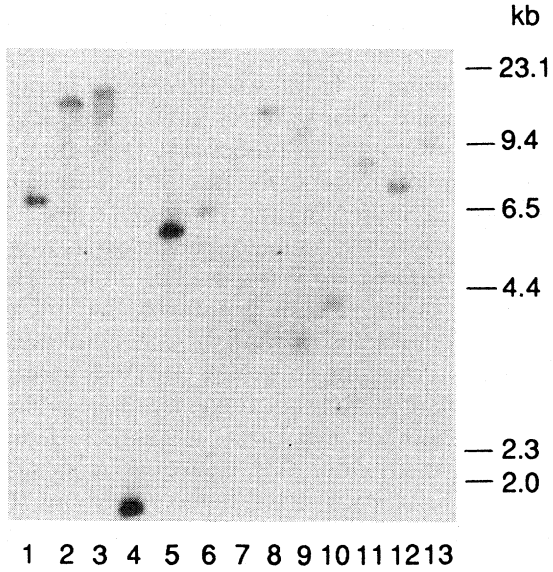

Fig. 3. Hybridization of the *P. aeruginosa algC* gene probe with *Hin*dIII-*Sst*I digested bacterial genomic DNA. Lanes are as in Fig. 2.

fragments (Fig. 3). Again, only a single hybridizing fragment was seen per group I strain and all fragments were of differing size. Weakly hybridizing fragments of varying sizes were seen for all other strains except for *P. cepacia* 376. As with the *algA* probe, *P. gladioli* was the only strain to give two hybridizing fragments (Fig. 3).

A 1.2 kb *Bam*HI-*Xho*I DNA fragment containing the *P. aeruginosa algD* gene was cut from plasmid pVD211 [12] and used as a probe for homologous sequences in *Bgl*II-*Cla*I digested genomic DNA that may encode for GMD enzyme activity. This fragment contains the *algD* promoter, the leader sequence and part of the *algD* coding region [12]. All five group I strains showed hybridizing fragments to the algD probe of varying sizes, with one fragment per strain (Fig. 4). For the representatives of the other rRNA groups, single, weakly-hybridizing fragments of similar size were seen for *P. testosteroni* ATCC 11996 and *P. avenae* VS-1.

A 0.65 kb *Xho*I-*Nsi*I DNA fragment containing the *P. aeruginosa algR1* (formerly *algR*) gene was cut from plasmid pKS30 [20] and used as a probe for homologous sequences in *Bgl*II digested genomic DNA that may encode for a

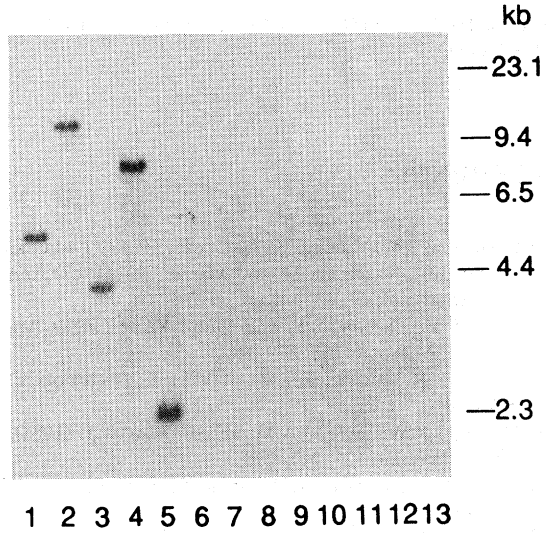

Fig. 4. Hybridization of the *P. aeruginosa algD* gene probe with *Bgl*II-*Cla*I digested bacterial genomic DNA. Lanes are as in Fig. 2.

positive regulatory protein. This fragment is internal to the *algR1* coding region (0.75 kb) [13]. With the *algR1* probe, single strongly hybridizing fragments of differing sizes were seen for all group I representatives with one fragment per strain (Fig. 5). All other strains did not contain hybridizing fragments.

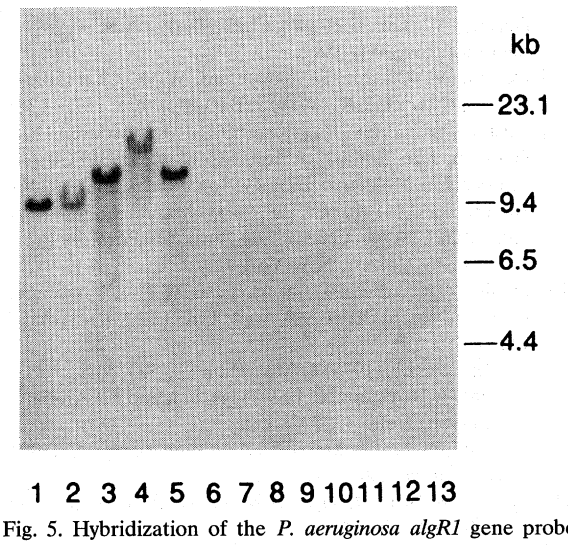

Fig. 5. Hybridization of the *P. aeruginosa algR1* gene probe with *Bgl*II digested bacterial genomic DNA. Lanes are as in Fig. 2.

5. DISCUSSION

The presence of highly homologous alginate gene sequences only in strains belonging to rRNA group I together with previous results on alginic acid production [2,4,21] suggest that the ability of pseudomonads to produce this exopolysaccharide is restricted to members of group I. Group I is further subdivided into subgroups Ia and Ib representing members incapable of producing or capable of producing fluorescent pigments, respectively [22]. The presence of highly homologous alginate gene fragments in *P. corrugata* strain 388, a representative of subgroup Ia, is of interest in light of our previous findings that a different strain of *P. corrugata* did not produce alginate under the single set of cultural conditions tested [21]. Alginate-producing variants of another member of subgroup Ia, *P. mendocina*, were generated by selecting for carbenicillin resistance, but this selection method was unsuccessful with subgroup Ia member *P. stutzeri* [4,23]. *P. stutzeri* was previously reported to contain sequences with homology to *algA, algC* and *algR1*, but not to *algD* [16]. However, Goldberg and Ohman [24,25] found that *algS (On) T* containing clones activated silent alginate genes *in trans* causing alginate production by *P. stutzeri*. The gene *algS* controls the expression of the adjacent trans-active regulatory gene *algT*. These findings imply that all members of subgroup Ia may be capable of alginate production and we are currently reassessing the ability of *P. corrugata* to produce alginate using a variety of culture conditions.

The variation in size of the hybridizing fragments seen among group I strains for all four alginate gene probes indicates that evolutionary divergence has occurred within this rRNA homology group. This divergence has occurred even within species as indicated by the differing sizes of hybridizing fragments exhibited by the three strains of *P. viridiflava* which originated from different plant hosts. The sizes of the hybridizing fragments found in this study for *P. syringae* pv. *glycinea* strain NCPPB 2159 were similar to those reported by Fiahlo et al. [16] except for *algR1*. The hybridizing fragment we detected in *Bgl*II

digested genomic DNA was 9.4 kb compared to the approximate 24-kb hybridizing fragment reported by Fiahlo et al. [16]. Possibly they did not obtain complete digestion of their genomic DNA for this strain.

Gene fragments with high homology to the four alginate genes used in this study were previously detected in several strains of the subgroup Ib pseudomonad *P. marginalis* [16]. Studies in our laboratory determined that under the in vitro conditions employed these strains produce novel acidic exopolysaccharides differing from alginic acid ([26, 27] Osman and Fett, unpublished). Thus, the alginate genes in these strains appear uninduced, defective or the strains are regulatory mutants.

The results of the present study lend further support for the considerable phylogenetic distance between members of pseudomonad rRNA group I and groups II–IV. Based on 16S rRNA sequencing and oligonucleotide cataloguing Woese and associates (for a review see [28]) assigned group I pseudomonads to the γ subdivision, γ-3 subgroup of the purple bacteria. Group III members *P. testosteroni* and *P. acidovorans* as well as *P. cepacia* were assigned to the β-subdivision of the purple bacteria in subgroups 1 and 2, respectively. Group IV member *P. diminuta* was assigned to α subdivision subgroup 2 of the purple bacteria. Based primarily on DNA-rRNA hybridization studies [29], it was proposed that the genus *Pseudomonas* be limited to group I members only since the different rRNA homology groups are more closely related to other genera than to each other. The process of assigning group II–IV pseudomonads to other genera has begun, as evidenced by the recent reclassification of *P. acidovorans* and *P. testosteroni* to the genus *Comamonas* [30] and of *P. delafieldii* and *P. facilis* (both group III) to *Acidovorax* [31].

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REFERENCES

[1] Costerton, J.W., Cheng, K.-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) Annu. Rev. Microbiol. 41, 435–464.
[2] Fett, W.F., Osman, S.F. and Dunn, M.F. (1989) Appl. Environ. Microbiol. 55, 579–583.
[3] Evans, L.R. and Linker, A. (1973) J. Bacteriol. 116, 915–924.
[4] Govan, J.R.W., Fyfe, J.A.M. and Jarman, T.J. (1981) J. Gen. Microbiol. 125, 217–220.
[5] Cote, G.L. and Krull, L.H. (1988) Carbohydr. Res. 181, 143–152.
[6] Pindar, D.F. and Bucke, C. (1975) Biochem. J. 152, 617–622.
[7] Gross, M. and Rudolph, K. (1987) J. Phytopathol. 118, 276–287.
[8] Gacesa, P. and Russell, N.J. (1990) In: *Pseudomonas* Infection and Alginates. (Gacesa, P. and Russell, N.J., Eds.), pp. 29–49. Chapman and Hall, New York, NY.
[9] Sanford, P.A. and Baird, J. (1983) In: The Polysaccharides Vol. 2, (Aspinall, G.O., Ed.) pp. 411–490 Academic Press, New York, NY.
[10] Skjak-Braek, G.H. and Larsen, B. (1986) Carbohydr. Res. 154, 239–250.
[11] May, T.B., Shinbarger, D., Maharaj, R., Kato, J., Chu, L., DeVault, J.D., Roychoudhury, S., Zielinski, N.A., Berry, A., Rothmel, R.K., Misra, T.K. and Chakrabarty, A.M. (1991) Clin. Microbiol. Rev. 4, 191–206.
[12] Deretic, V., Gill, J.F. and Chakrabarty, A.M. (1987) J. Bacteriol. 169, 351–358.
[13] Deretic, V., Dikshit, R., Konyecsni, W.M., Chakrabarty, A.M. and Misra, T.K. (1989) J. Bacteriol. 171, 1278–1283.
[14] Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1991) Current Protocols in Molecular Biology. John Wiley & Sons, NY.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[16] Fiahlo, A.M., Zielinski, N.A., Fett, W.F., Chakrabarty, A.M. and Berry, A. (1990) Appl. Environ. Microbiol. 56, 436–443.
[17] Southern, E.M. (1975) J. Mol. Biol. 98, 503–517.
[18] Darzins, A.B., Frantz, R.I., Vanags, R.I. and Chakrabarty, A.M. (1986) Gene 42, 293–302.
[19] Zielinski, N.A., Chakrabarty, A.M. and Berry, A. (1991) J. Biol. Chem. 266, 9754–9763.
[20] Kato, J. and Chakrabarty, A.M. (1991) Proc. Natl. Acad. Sci. USA 88, 1760–1764.
[21] Fett, W.F., Osman, S.F., Fishman, M.L. and Siebles, T.S., III. (1986) Appl. Environ. Microbiol. 52, 466–473.

[22] Palleroni, N.J. (1984) In: Bergey's Manual of Systematic Bacteriology, Vol. 1, (Kreig, N.R. and Holt, J.G., Eds.), pp. 141–199. The Williams and Wilkins Co., Baltimore, MD.
[23] Hacking, A.J., Taylor, I.W.F., Jarman, T.R. and Govan, J.R.W. (1983) J. Gen. Microbiol. 129, 3473–3480.
[24] Goldberg, J. and Ohman, D.E. (1989) Proc. 1989 Annu. Meeting Amer. Soc. Microbiol. 84.
[25] Goldberg, J. (1992) Harvard University. Personal communication.
[26] Osman, S.F. and Fett, W.F. (1989) J. Bacteriol. 171, 1760–1762.
[27] Osman, S.F. and Fett, W.F. (1990) Carbohydr. Res. 199, 77–82.
[28] Woese, C.R. (1987) Microbiol. Rev. 51, 221–271.
[29] DeVos, P., Van Landschoot, A., Segers, P., Tytgat, R., Gillis, M., Bauwens, M., Rossau, R., Goor, M., Pot, B., Kersters, K., Lizzaraga, P. and DeLey, J. (1989) Int. J. Syst. Bacteriol. 39, 35–49.
[30] Tamaoka, J., Ha, D.M. and Komagata, K. (1987) Int. J. System. Bacteriol. 37, 52–59.
[31] Willems, A., Falsen, E., Pot, B., Jantzen, E., Hoste, B., VanDamme, P., Gillis, M., Kersters, K. and DeLey, J. (1990) Int. J. System. Bacteriol. 40, 384–398.
[32] DeVos, P., Goor, M., Gillis, M. and DeLay, J. (1985) Int. J. Syst. Bacteriol. 35, 169–183.